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Effects of a Supplement Containing Apoaequorin on Verbal Learning in Older Adults in the Community

Daniel L. Moran, PhD; Mark Y. Underwood, BA; Taylor A. Gabourie, BS; Kenneth C. Lerner, MS, MBA

ABSTRACT

Context • The changes in verbal learning and working memory that often occur with aging may result in reduced social and intellectual interactions. These changes significantly affect an individual's quality of life. As humans age, the body's ability to regulate and maintain calcium levels is diminished. Pharmacological manipulation of the entry of free calcium (Ca^{2+}) has been shown to be effective in increasing some aspects of cognitive function in the aged brain. Apoaequorin has been shown in laboratory studies to regulate levels of intracellular calcium in neuronal cells and to provide protection against ischemic cell death.
Objective • The study was designed to assess the effects of a supplement of apoaequorin on verbal learning and working memory.
Design • The current study, the Madison Memory Study, was a randomized, double-blind, placebo-controlled trial.
Setting • The study occurred in Madison, WI, USA.
Participants • Participants were 218 community-dwelling adults, aged 40-91 y, with self-reported memory concerns.
Intervention • Participants were randomly assigned to receive either apoaequorin (apoaequorin group) or a matched placebo (control group) for 90 d.
Outcome Measures • The study used quantitative, computerized tools for cognitive assessment the CogState International Shopping List (ISL) and the CogState ISL-Delayed Recall (ISL-DR). Scores from computerized cognitive tasks were measured at baseline and at several points during the 90-d study.
Results • No significant differences existed between the intervention and control groups in any parameter at baseline. The intervention group (apoaequorin group) showed a statistically significant improvement in verbal learning and recall on the ISL and the ISL-DR, respectively, during the 90-d study. Apoaequorin was tolerated very well in the study.
Conclusions • The results indicated a strong relationship between apoaequorin and improvements on a quantitative measure of cognitive function, specifically verbal learning. The study found that apoaequorin is a well-tolerated supplement that improved cognitive function in aging adults. The results suggest potential utility for apoaequorin in addressing the declines in cognitive function associated with aging. (*Adv Mind Body Med.* 2016;30(1):4-11.)

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Every 7 seconds in the United States, another member of the Baby Boomer generation turns 65 years old. As of 2012, 810 million people were older than 60 years worldwide, accounting for 11% of the world population. That number of people is expected to rise to 2 billion by the year 2050, or 22% of the world population.[1]

Normal cognitive function is central to many people's quality of life. Verbal learning is one of the most common ways an individual interacts with his or her environment. Verbal learning is the ability to hear information and retain it for later use. Examples of verbal learning include tasks such as remembering a list of items or recalling the name of a person whom an individual has recently met. Although those abilities are routine for many adults, studies have shown that

verbal learning declines with age.[2] An inability to recall new information can significantly affect the quality of life for older adults.[3]

Role of Calcium

Calcium has many functions throughout the body. It is an essential second messenger that plays important roles in a plethora of neuronal functions, including synaptic plasticity, activation of kinases and phosphatases, regulation of gene expression, and excitotoxic cell-death plasticity.[4,5,6,7,8] Calcium-dependent processes have been shown to be important for associative learning in both adult and aged animals.[9,10,11]

Although calcium is vital to normal neuronal function, too much calcium is detrimental. Calcium levels in the nervous system are tightly regulated. During normal physiological activity, a brief elevation of seconds to minutes occurs in the concentration of intracellular calcium. The short-lived increase quickly returns to baseline without any adverse effects on the neuron.

As humans age, the body's ability to regulate and maintain calcium levels closely is reduced. Elevated calcium levels have frequently been noted in older neurons, and aging nervous systems exhibit an increased sensitivity to changes in calcium levels. When aged neurons are activated, they experience excessive calcium influx, particularly neurons in the brain regions that are vital for normal memory, such as the hippocampus and the associated structures of the medial temporal lobe.[10,12,13] Aging neurons are less able to maintain calcium homeostasis, due at least partly to decreases in calcium-binding proteins, which help to buffer intracellular calcium.[14] An inability to maintain appropriate levels of intracellular calcium is believed to be involved in brain aging.

Pharmacological manipulation of the entry of free calcium (Ca^{2+}) has been shown to be effective in increasing some aspects of cognitive function in the aged brain. Compounds that block the influx of calcium through L-type calcium channels have been shown to both improve associative learning in aged animals[15,16,17] and restore the electrophysiological properties to those commonly seen in young adults.[12,18,19]

The changes that researchers have seen when cells are unable to regulate calcium levels gave rise to the calcium hypothesis of brain aging. First proposed by Khachaturian,[20,21,22] the calcium hypothesis was based on effects noted in relation to elevated calcium levels in neuronal cells. Khachaturian had postulated that small increases in the levels of intracellular calcium, resulting from a dysregulation of calcium homeostasis, could lead in time to damage similar to that seen after an acute rise in calcium levels, eventually leading to cell death. Many investigators have postulated that sustained alterations in calcium levels provide a final, common pathway for age-associated alterations in brain function.[23] Further explorations of Ca^{2+} homeostasis, regulation, and signaling might reveal the mechanisms that are involved in the age-dependent decline in neuronal performance and might aid the search for new therapeutic treatments.[24,25]

Apoaequorin

Apoaequorin is a naturally occurring, calcium-binding protein, originally from a species of jellyfish,[26] that is available commercially as a dietary supplement. Apoaequorin has been determined to be safe[27] and nonallergenic,[28] with an amino-acid sequence similar to human calcium-binding proteins. Calcium-binding proteins have been shown to be vital to the regulation of calcium levels in certain cell types, with lower levels present in aged cells. Cells that exhibit calcium dyshomeostasis have decreased levels of calcium-binding proteins.[29]

A possible strategy for managing altered calcium levels in neuronal cells involves supplementation with exogenous calcium-binding proteins. Apoaequorin has been shown in laboratory studies to regulate levels of intracellular calcium in neuronal cells and to provide protection against ischemic cell death.[30,31] Based on the similarity of its sequence and in vitro and in vivo results,[32] the current research team hypothesized that apoaequorin has the potential to improve the function of aging neurons and, therefore, to enhance memory and cognitive function.

Increasing the levels of calcium-binding proteins has been shown to restore more appropriate regulation of intracellular levels of calcium. Previous work with apoaequorin in cognitive trials with aged canines has demonstrated cognitive enhancement compared with the effects that were seen in the control group.[33]

The primary objective of the current study, the Madison Memory Study, was to assess the effects of apoaequorin on cognitive function. Quantitative, computerized cognitive tests were employed to examine the effects of apoaequorin in time and compared with placebo.

METHODS

The study was a randomized, double-blind, placebo-controlled trial designed to examine the effects of apoaequorin on cognitive function in older adults.

Participants

The Madison Memory Study took place in Madison, WI, USA. Participants were community-dwelling individuals from Madison and surrounding communities in Dane County, WI, USA. Potential participants were recruited via advertisements in local publications and informational flyers posted in a variety of local venues. Potential participants who expressed an interest completed an initial health screening and demographic questionnaire to determine eligibility for the Madison Memory Study. Those who met the inclusion criteria and were not excluded by the exclusion criteria were assigned a unique study subject number (SSN) upon their acceptance into the Madison Memory Study. SSNs were randomly assigned to either the control or treatment arms. Participants, study coordinators, and evaluators remained blinded until the end of the study. Study supply containers were labeled with the participants' SSNs. Each participant gave written consent prior to baseline testing. Participants

were encouraged to inform their study coordinator of any adverse events; these were recorded by the study coordinator.

To be included, prospective participants needed to (1) be a healthy male or female; (2) be between 40 and 95 years of age at baseline; (3) have concerns related to memory issues; and (4) be able to comply with the study's protocol and to complete periodic, computerized, cognitive tests. Exclusion criteria included (1) a history of significant neurological disease, dementia, or related memory-impairment disorders or an untreated psychotic or major depressive disorder; (2) a history of poorly controlled hypertension, uncontrolled or insulin-dependent diabetes, or other uncontrolled medical condition; (3) a history of hypersensitivity to any of the components of the test materials for the intervention or control group; or (4) an individual, in the investigator's opinion, who was unlikely to comply with the study protocol and/or complete periodic, computerized, cognitive testing.

Procedures

All participants completed the AD8 screening interview (AD8) prior to baseline testing. To segregate participants by their levels of self-reported cognitive impairment, participants' baseline scores on the AD8 were acquired. The AD8 is a brief, 8-question, reliable screening tool that is sensitive and predictive in classifying nondemented or cognitively normal, older adults from those with some level of cognitive impairment.[34,35]

Informant AD8 scores are generally preferred to participant AD8 scores, because they have been shown to be more strongly correlated with accurate differentiation between demented and nondemented individuals. The availability of an informant was not an inclusion criterion for the study and, therefore, participant AD8 scores were used.[35] An AD8 score of 2 was used as a cut-off value to discriminate between cognitively normal individuals and those with some level of cognitive impairment.[36] Table 1 shows the results of the AD8 testing.

Participants were required to complete 5 computerized, cognitive testing sessions. Computerized tasks were selected from the CogState Research Battery (CogState Ltd, Melbourne, Victoria, Australia). The CogState International Shopping List (ISL) and the ISL-Delayed Recall (ISL-DR) tasks were administered to participants following a script provided by CogState Ltd at predetermined testing intervals (ie, day 0-baseline, day 8, day 30, day 60, and day 90 [study completion]). The primary efficacy variable was the change in performance on the tasks from baseline, day 0, to the end of the study, day 90.

Table 1. Distribution of Participants Within AD8 Subgroups

	AD8 Scores	
Group	0-2	2-5
Control group	38	56
Apoaequorin group	59	68

Abbreviation: AD8, AD8 screening interview.

Capsules of the dietary supplement were provided by Quincy Bioscience (Madison, WI, USA) and were manufactured according to current Good Manufacturing Practice (GMP) regulations and standards in the normal course of the company's manufacturing. The quantification of the composition and the purity of the protein were confirmed through the company's standard operating procedures, which included an analysis using a bicinchoninic acid assay (BCA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Results were within specifications.

Microbiological and moisture testing were conducted by the Minnesota Valley Testing Laboratories (New Ulm, MN, USA). Microbiological testing included a coliform count and tests for *Escherichia coli*, *Staphylococci aureus*, mold, yeast, and salmonella. The testing did not detect the presence of any microorganisms in the capsules that were used in testing.

Intervention

Participants in the control group received capsules containing only white-rice flour. Participants in the apoaequorin group received capsules containing 10 mg of oral apoaequorin and white-rice flour. Capsules were size and color matched.

Participants were instructed to take 1 capsule daily for the duration of the study. The dosage of the apoaequorin was selected because of its use and availability as a marketed dietary supplement. Daily administration was chosen because of previous work demonstrating the period of availability for apoaequorin in tested hippocampal cells.[31]

Outcome Measures

The cognitive measures used in the current study are part of the CogState Research Test Battery and have been described previously elsewhere.[37,38]

CogState is a widely used battery of computerized cognitive tests that are adaptations of standard neuropsychological tests. CogState was selected because its tasks are brief and repeatable and have shown little or no practice effects,[39] thus having utility for the repeated assessment of cognition in older adults. Practice sessions were presented before each task to ensure that a participant was aware of the rules for each task.

The CogState tasks used in this study included the ISL and the ISL-DR, which measure changes in verbal learning and working memory.[39,40] Verbal learning is the cognitive function associated with memorization and the retention of a list of words. However, verbal learning does not consist solely of memorization. It refers to the ability to learn information verbally.[2] Verbal working memory is the ability to keep instructions in working memory and use them to perform a task. The ability to use verbal working memory is necessary to perform a task that is preceded by verbal instructions.

International Shopping List. The ISL is a 12-word, 3-trial test of verbal list learning that is similar to other verbal list assessments. In the ISL, the presentation of stimuli and the recording of responses are facilitated by a trained proctor

Table 2. Characteristics of Participants in Subgroups

Group	n	Age Range	Mean ± SD
All participants	218	40 to 91	62.48 ± 11.43
Male	70		
Female	148		
Apoaequorin group	126	40 to 90	62.54 ± 11.79
Male	43		
Female	83		
Control group	92	40 to 91	62.39 ± 10.98
Male	27		
Female	65		

Abbreviation: SD, standard deviation.

and recorded by a computer. Each 12-word list that is used is generated by the software and presented in a random order. The list is presented 3 times to the participants. The ISL has good sensitivity to impaired or altered verbal memory.[41,42] The primary outcome measure for the ISL is the change in the total number of shopping list words that participants are able to remember during the 3 iterations of the ISL.

ISL-Delayed Recall. The ISL-DR measures verbal learning and delayed memory or recall. The primary outcome measure for the ISL-DR is the number of words that a participant can remember from the shopping list that he or she had seen approximately 25 minutes earlier in the 3 trials for the ISL.

Statistical Analysis

Data were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) Version 19 (IBM, Inc, Armonk, NY, USA). Data from the CogState testing were analyzed as recommended by CogState Ltd and by previous studies.[2,43,44,45] Briefly, cognitive assessments were analyzed using paired and independent *t* tests and the mixed-model repeated measures analysis of covariance (ANCOVA). The scores at baseline served as the covariate for the mixed-model repeated measure ANCOVA.

In addition to an analysis of the study's entire population, the current research team segregated participants into analysis groups based on self-reported levels of cognitive impairment, as measured by the AD8. Qualitative survey data were analyzed to determine descriptive statistics, using the Mann-Whitney *U* test and the Wilcoxon signed-rank test. Group means and standard deviations were found for each cognitive assessment.

Effect size is a statistical measure that describes the strength or magnitude of the difference between 2 groups or between time points. Effect size is calculated by taking the difference in the means of the 2 groups or time points and dividing that number by the pooled or combined standard deviation. The value of the effect size measures the strength of the observed effect between the 2 groups or time points.

The effect size for Cohen's *d* is measured on a standardized scale starting at 0.0. It examines the relationship between 2 groups to determine the degree of difference between scores. A Cohen's *d* of 0.25 indicates a weak effect for the experimental variable in relation to the variability in the sample from baseline to postintervention. An effect size of 0.2 is considered a small effect; an effect size of 0.5 is considered a medium effect; and an effect size of 0.8 or greater is considered a large effect.

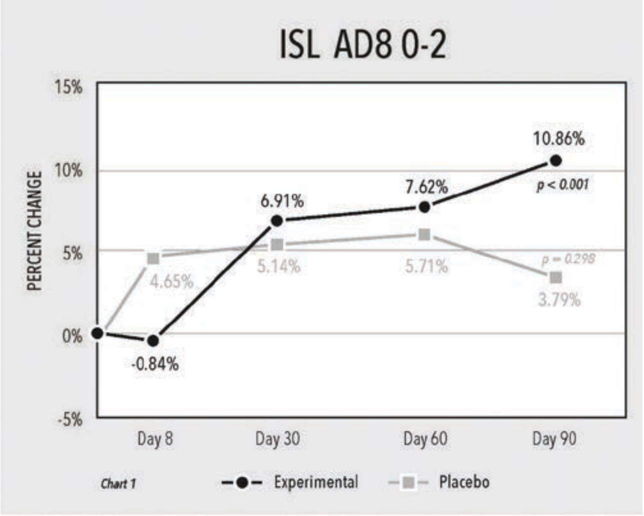
RESULTS

The Madison Memory Study included 218 participants, 148 females and 70 males, aged 40 to 91 years, with a mean age of 62.48 ± 11.43 years. Table 2 shows the demographics of the 2 groups.

International Shopping List

Postintervention, a significant difference in the number of items correctly recalled on the ISL was not seen between the control and apoaequorin groups when the study's entire population was analyzed. However, when the results were analyzed for the study's participants with AD8 scores from 0 to 2 (ie, individuals who were cognitively normal), a statistically significant difference was found in the number of items correctly recalled between baseline and postintervention time points for participants in the apoaequorin group that was not present in the control group. Examining the number of correct responses, the apoaequorin group showed a 10.86% increase in the number of items correctly recalled, with $t_{56} = 4.40$, $P < .001$ (Figure 1). The control group exhibited a 3.79% improvement in the number of items recalled, with $t_{32} = 1.059$, $P = .298$. The effect size for the apoaequorin group postintervention was 1.63, indicating a large effect (Figure 2).

Figure 1. Apoaequorin (Experimental) and Control (Placebo) Groups



Note: The change in the number of items recalled on the ISL from baseline to postintervention for participants with baseline scores on the AD8 from 0 to 2.

Abbreviations: ISL, International Shopping List; AD8, AD8 screening interview.

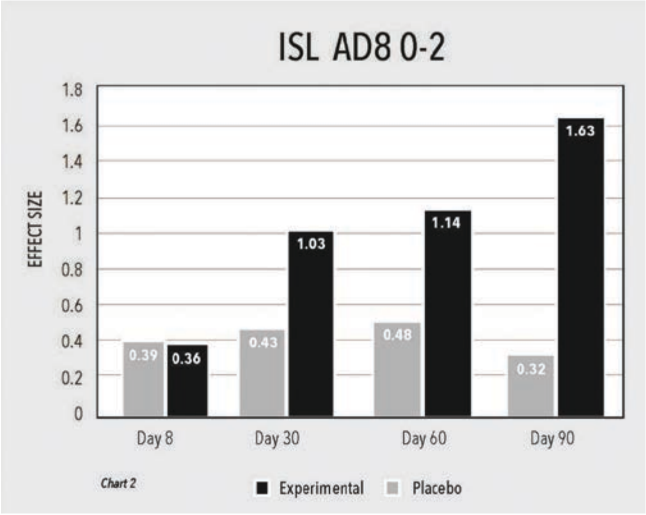
ISL-Delayed Recall

Postintervention, a significant difference was seen in the apoaequorin group ($P = .044$) in the number of items correctly recalled that was not present in the control group when the data for the study's complete population were analyzed. The apoaequorin group exhibited an effect size of 0.31, indicating a small effect for the intervention for the study's complete population. The apoaequorin group also showed a larger percentage of participants with improvements in their scores at postintervention versus baseline when compared with participants in the control group.

When the study's participants with AD8 scores of 0 to 2 were analyzed, a significant difference was seen between the number of items recalled for participants in the apoaequorin group, with $t_{56} = 3.010$, $P = .004$ at day 90 compared with baseline. A significant difference was not seen for participants in the control group from baseline to postintervention, with $t_{32} = 1.202$, $P = .238$. The apoaequorin group demonstrated a 15.82% increase in the number of items correctly recalled (Figure 3). The control group showed a 7.41% increase in the number of items recalled. The apoaequorin group exhibited an effect size of 0.78, indicating a moderate or large effect (Figure 4).

The performance improvement on the ISL-DR was also noted when an additional subset of the study's population was segregated for analysis. For participants with AD8 scores between 2 and 5, indicating greater self-reported, cognitive

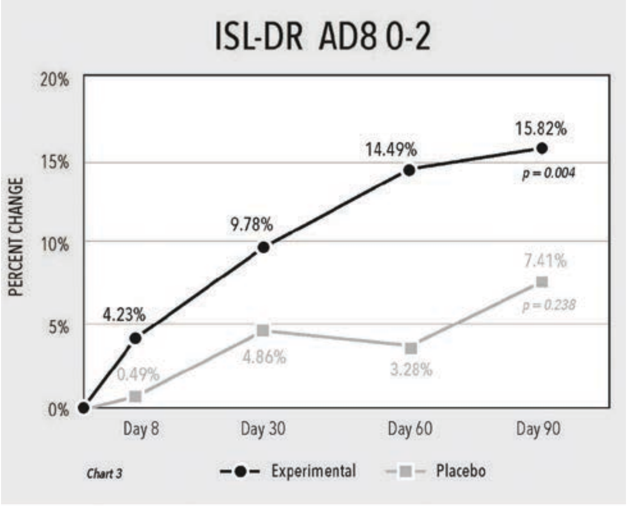
Figure 2. Apoaequorin (Experimental) and Control (Placebo) Groups



Note: Effect sizes for the change in the number of items recalled on the ISL from baseline to postintervention for participants with baseline scores on the AD8 from 0 to 2. The apoaequorin group showed a large effect.

Abbreviations: ISL, International Shopping List; AD8, AD8 screening interview.

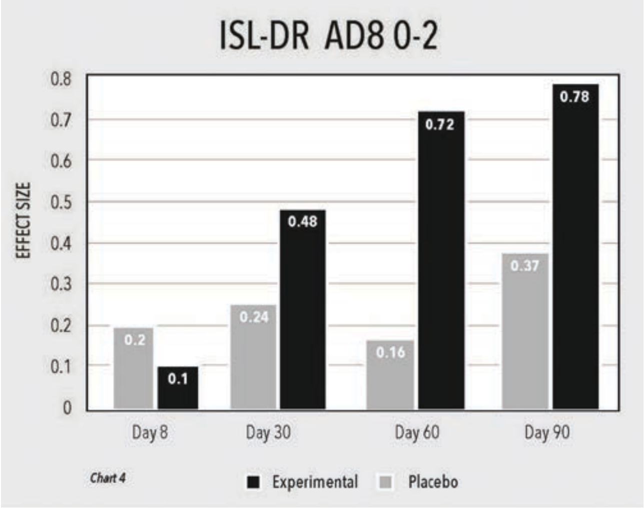
Figure 3. Apoaequorin (Experimental) and Control (Placebo) Groups



Note: The change in the number of items recalled on the ISL-DR from baseline to postintervention for participants with baseline scores on the AD8 from 0 to 2.

Abbreviations: ISL-DR, International Shopping List-Delayed Recall; AD8, AD8 screening interview.

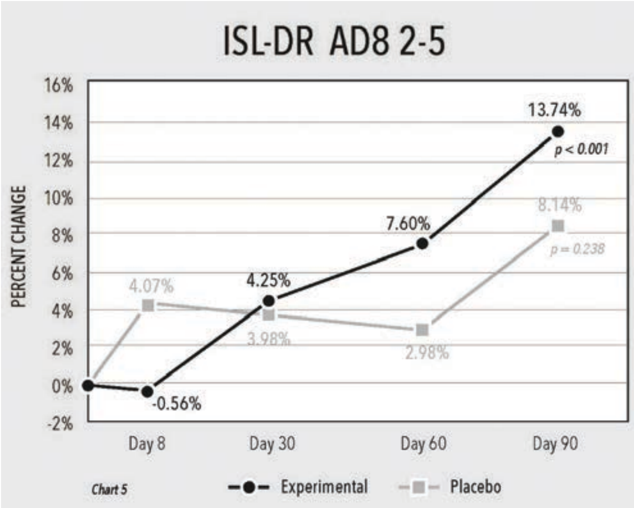
Figure 4. Apoaequorin (Experimental) and Control (Placebo) Groups



Note: Effect sizes for the change in the number of items recalled on the ISL-DR from baseline to postintervention for participants with baseline scores on the AD8 from 0 to 2. The apoaequorin group showed a moderate to large effect.

Abbreviations: ISL-DR, International Shopping List-Delayed Recall; AD8, AD8 screening interview.

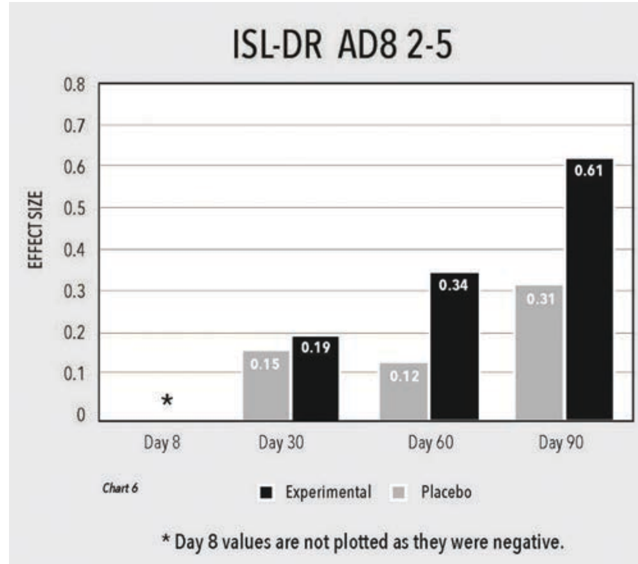
Figure 5. Apoaequorin (Experimental) and Control (Placebo) Groups



Note: The change in the number of items recalled on the ISL-DR from baseline to postintervention for participants with baseline scores on the AD8 from 2 to 5.

Abbreviations: ISL-DR, International Shopping List-Delayed Recall; AD8, AD8 screening interview.

Figure 6. Apoaequorin (Experimental) and Control (Placebo) Groups



Note: Effect sizes for the change in the number of items recalled on the ISL-DR from baseline to postintervention for participants with baseline scores on the AD8 from 2 to 5. The apoaequorin group showed a medium effect.

Abbreviations: ISL-DR, International Shopping List-Delayed Recall; AD8, AD8 screening interview.

difficulties, the apoaequorin group, with $t_{42} = 4.913$ and $P < .001$, showed a statistically significant improvement that was not present in the control group, with $t_{36} = 1.423$ and $P = .163$ (Figure 5). The effect size for the apoaequorin group was 0.61, indicating a medium effect (Figure 6).

Adverse Events

The intervention and control substances were tolerated very well. Two participants experienced adverse events during the study. Each group had a single adverse event, and no serious adverse events (SAEs) occurred in the study.

The participant in the apoaequorin group reported "feeling owly" or irritable. The participant in the control group reported that the test article made him feel despondent and tired all of the time. Neither participant sought or received any medical intervention associated with the adverse events. Both participants withdrew from the study.

DISCUSSION

Many studies have examined the effects of a wide range of interventions focused on maintaining or improving cognitive function. The current study was designed to examine the effects of oral supplementation of the calcium-binding protein apoaequorin on cognitive function in a population of community-dwelling, older adults with self-reported cognitive difficulties or concerns. The randomized, double-blind, placebo controlled study was performed to validate anecdotal reports of improvements in cognitive function after use of a dietary supplement of oral apoaequorin. Changes in cognitive function were quantitatively assessed using tasks from the CogState Research Battery.

The current study's data showed statistically significant changes in verbal learning and working memory in the apoaequorin group for the study's complete population and for a subset of that population with little or no self-reported cognitive issues (ie, those having an AD8 of 0-2). Those data support the hypothesis that oral supplementation with the calcium-binding protein apoaequorin can affect cognitive function positively.

The improvements seen for that subset of the study's population, with no or minimal self-reported cognitive difficulties, showed a Cohen's *d* effect size in the medium to large range, further indicating that the intervention substance can provide meaningful changes in cognitive function to the population.

It is highly unlikely that the results seen on the ISL and ISL-DR tasks were due to chance. The differences seen in the apoaequorin group with an AD8 = 0 to 2 for the ISL and ISL-DR were not trivial but represent a difference of greater than 10% or 15%, respectively, from baseline scores. The differences between the values at baseline and postintervention for the apoaequorin group were also highly significant statistically, whereas no significance was seen for the control group.

The strengths of the Madison Memory Study included the recruitment of a community-dwelling population and the use of quantitative cognitive testing. The CogState Research Battery provided the ability to quantify subtle changes in cognitive performance in a population of older individuals with varying degrees of cognitive impairment, without learning or ceiling effects.[38,43]

The current study had several limitations. The study did not attempt to control for a number of variables that may have affected participants' performance on the computerized cognitive tests. Uncontrolled variables include, but are not limited to, coexistent medications and medical or psychiatric conditions that did not meet the threshold for exclusion.

Participants' adherence to daily dosing was also a limitation in the study. Although participants were queried regarding compliance with daily dosing, strict compliance with the dosing regimen could not be adequately controlled in the community-based study.

In addition, participants were enrolled who had a range of self-reported memory issues, as measured by participants' AD8 scores. Although the desired number of participants was enrolled, their dispersal across the range of AD8 scores reduced the statistical power of the study. Limiting enrollment to individuals with AD8 scores between 0 and 2 would provide for a more homogenous population with minimal or no self-reported memory complaints.[35]

Also, the participants in the current study were from a population that had self-reported some memory issue or age-associated change in cognitive function. Although an AD8 cutoff of less than 2 is commonly used to discriminate between cognitively normal individuals and those with some cognitive issues, an AD8 score of 2 was used as the cut-off value to include both cognitively normal participants and those with minimal, self-reported memory issues.

The current research team believes that additional studies with more defined populations (ie, a population with AD8 scores from 0-2) would provide even clearer evidence for the ability of the calcium-binding protein apoaequorin to affect cognitive function positively in aged individuals with minimal or no cognitive impairment. With regard to the statistically significant results seen for populations with greater self-reported cognitive impairment (ie, with an AD8 > 2 at baseline), a larger study and a longer time frame may provide additional evidence of a beneficial effect in that particular population.

CONCLUSION

Apoaequorin is a well-tolerated, calcium-binding protein that has demonstrated the ability to improve cognitive function. In the current study, daily use of an oral apoaequorin-containing supplement or capsule had a statistically significant effect on verbal learning in a population of older individuals with little or no self-reported cognitive impairment. Those results indicated a strong relationship between apoaequorin and improvements on a measure of cognitive function, specifically verbal learning. The results imply possible utility for apoaequorin to reduce the declines in cognitive function associated with aging.

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